BIOCHEMISTRY
including biophysical chemistry & molecular biology

Article

pubs.acs.org/biochemistry

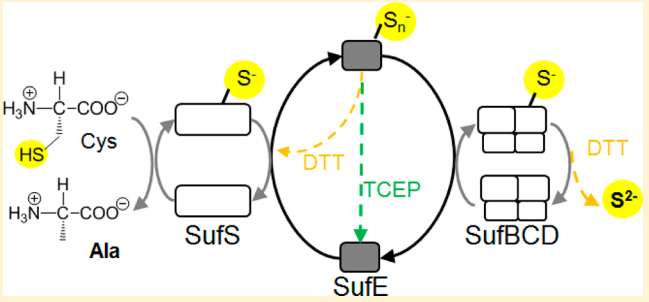
Protected Sulfur Transfer Reactions by the *Escherichia coli* Suf System

Bruna P. Selbach, Pradyumna K. Pradhan, and Patricia C. Dos Santos*

Department of Chemistry, Wake Forest University, Winston-Salem, North Carolina 27109, United States

Supporting Information

ABSTRACT: The first step in sulfur mobilization for the biosynthesis of Fe−S clusters under oxidative stress and iron starvation in *Escherichia coli* involves a cysteine desulfurase SufS. Its catalytic reactivity is dependent on the presence of a sulfur acceptor protein, SufE, which acts as the preferred substrate for this enzyme. Kinetic analysis of the cysteine:SufE sulfurtransferase reaction of the *E. coli* SufS that is partially protected from reducing agents, such as dithiothreitol and glutathione, was conducted. Under these conditions, the reaction displays a biphasic profile in which the first phase involves a fast sulfur transfer reaction from SufS to SufE. The accumulation of persulfurated/polysulfurated forms of SufE accounts for a second phase of the slow catalytic turnover rate. The presence of the SufBCD complex enhances the activity associated with the second phase, while modestly inhibiting the activity associated with the initial sulfur transfer from SufS to SufE. Thus, the rate of sulfur transfer from SufS to the final proposed SufBCD Fe−S cluster scaffold appears to be dependent on the availability of the final sulfur acceptor. The use of a stronger reducing agent [tris(2-carboxyethyl)phosphine hydrochloride] elicited the maximal activity of the SufS−SufE reaction and surpassed the stimulatory effect of SufBCD. This concerted sulfur trafficking path involving sequential transfer from SufS to SufE to SufBCD guarantees the protection of intermediates at a controlled flux to meet cellular demands encountered under conditions detrimental to thiol chemistry and Fe−S cluster metabolism.



In bacterial and eukaryotic systems, the first step of sulfur incorporation in the biosynthesis of thio cofactors involves a pyridoxal 5′-phosphate (PLP) enzymatic mechanism. Cysteine desulfurases constitute a family of enzymes responsible for the sulfur transfer from cysteine to acceptor molecules.[1,2] The overall reaction can be divided into two discrete steps: (1) cleavage of the C−S bond and S activation through formation of a persulfide bond and (2) subsequent persulfide sulfur transfer to an acceptor molecule. In the first step, the cysteine substrate binds to the PLP cofactor at the enzyme's active site, forming a PLP−Cys external aldimine intermediate. This event is followed by activation of the amino acid substrate for the nucleophilic attack by an active site cysteine thiolate group. The cleavage of the C−S bond of the amino acid substrate leads to the formation of a persulfide enzyme intermediate and the release of alanine as the first product.[3]

While it is assumed that all cysteine desulfurases follow similar chemical steps in the first half of this catalytic cycle, the second half of the reaction, sulfur transfer to a specific acceptor molecule, is the committed step that dictates its biochemical function. Genetic and biochemical studies have identified dedicated sulfur acceptor proteins that are the physiological substrates of cysteine desulfurases.[4,5] These molecules, in their sulfurated forms, serve specific S entry points to pathways involving the synthesis of thio cofactors such as Fe−S clusters, thiamin, molybdenum cofactor, 4-thiouridine, and 2-thiouridine tRNA.[5] Although detailed spectroscopic studies have been performed for the first half of the reaction, most reports disregard the involvement of physiological sulfur acceptors and include reducing agents that act as artificial/competitor sulfur acceptors.

In *Escherichia coli*, three cysteine desulfurases have been identified: IscS, SufS, and CsdA. Functional and structural peculiarities among these enzymes allowed their classification into two mechanistically distinct groups.[6] IscS, a member of the class I cysteine desulfurases, performs a leading role in sulfur mobilization. In the biosynthesis of Fe−S clusters, IscS interacts with IscU, which serves as the platform for the assembly and delivery of inorganic Fe−S cofactors. In addition, IscS interacts with a suite of sulfur acceptor molecules for the biosynthesis of most, if not all, thio cofactors.[1] *In vitro* biochemical experiments showed that under reducing conditions, the presence of physiological acceptor proteins elicits little to no effect on IscS activity.[7,8] The promiscuous behavior of IscS with a variety of sulfur acceptor molecules is partially explained by the structural location of the active site cysteine. The residue carrying the persulfide sulfur is located in a long structurally disordered loop, which is thought to facilitate interactions with a variety of sulfur acceptor molecules.

Despite general similarities in their overall fold, quaternary structure, and reactivity toward cysteine, discrete differences among cysteine desulfurase sequences and structures allow

Received: February 6, 2013
Revised: April 25, 2013
Published: May 14, 2013

   dx.doi.org/10.1021/bi4001479 | *Biochemistry* 2013, 52, 4089−4096

classification of SufS and CsdA in a separate subgroup apart from IscS. As members of class II cysteine desulfurases, the active site cysteine residue of these enzymes is located a short structurally defined loop.[9,10] Their reactivity is dependent on the participation of a second protein that is proposed to act as their sole sulfur acceptor. In the case of SufS and CsdA, genes encoding the physiological S acceptors (*sufE* and *csdE*) are located immediately downstream of the cysteine desulfurase coding sequences. The corequirement of cysteine desulfurases and sulfur acceptors has been shown to be crucial for their cellular functions.[11,12] Although it appears that CsdA and CsdE have a supporting role in Fe−S cluster metabolism, SufS and SufE are major players in Fe−S cluster biogenesis under conditions of oxidative stress and iron starvation. Interestingly, the overexpression of CsdA but not the CsdA−CsdE pair can partially replace the function of SufS,[12] suggesting that CsdA is able to positively interact with the Suf system, possibly through direct interaction with SufE. In this system, SufE and SufBCD, the proposed Fe−S scaffold complex of the Suf system, cannot be substituted with any other sulfur acceptor or Fe−S cluster scaffold, thus revealing their unprecedented functions in Fe−S metabolism.

Preliminary kinetic analysis indicates that the catalytic turnover rate of the SufS cysteine desulfurase reaction is enhanced up to 50-fold in the presence of SufE.[8,13] Labeling experiments showed that residue Cys 51 of SufE can be modified by one to four covalently bound sulfur atoms.[14] In addition, the presence of the SufBCD complex further enhances the rate of sulfide production up to 30-fold in reaction mixtures containing SufS and SufE,[11] where up to seven sulfur atoms can accumulate in SufB.[15] A directed sulfur transfer model involving the sequential transfer of sulfur from SufS to SufE to SufB is further supported by affinity chromatography experiments that showed the direct interaction between SufE and SufS, and between SufE and SufBCD. Although there is compelling evidence for the general route of sulfur transfer used by the *E. coli* Suf system, neither the kinetic schemes of these reactions nor the mechanism of SufS enhancement has been resolved.

Our group has recently characterized the sulfurtransferase reaction catalyzed by the *Bacillus subtilis* class II cysteine desulfurase, SufS, and its sulfur acceptor substrate, SufU.[16] Initial velocity studies indicated a double-displacement mechanism for the cysteine:SufU sulfurtransferase reaction. The proposed scheme involved the formation of a covalently modified SufS and release of alanine preceding the binding of SufU and subsequent sulfur transfer reaction. The structural similarities between *E. coli* and *B. subtilis* SufS proteins and their dependencies on mutually devoted sulfur trafficking reactions prompted our investigation of the kinetic mechanism of sulfur transfer between *E. coli* SufS and SufE.

Here we describe the kinetic investigation of the cysteine:SufE sulfurtransferase reaction that is partially protected from the action of reducing agents such as dithiothreitol (DTT) and glutathione. The rate of sulfur trafficking from SufS to the final proposed SufBCD Fe−S cluster scaffold appears to be dependent on the availability of the final sulfur acceptor. Moreover, the use of a strong reducing agent [tris(2-carboxyethyl)phosphine hydrochloride (TCEP)] optimized the participation of the SufE sulfurtransferase substrate, enhancing up to 100-fold the rate of alanine formation. Kinetic evidence of the involvement of the SufS system in trafficking sulfur in a sequential protected route provides further support for its recruitment under conditions detrimental to thiol chemistry and Fe−S cluster assembly.

MATERIALS AND METHODS

Media and Chemicals. Reagents and chemicals were purchased from Fisher Scientific and Sigma-Aldrich Inc. unless specified. Cells were grown on commercially available Lennox Broth (LB) medium with the desired antibiotics [ampicillin (100 μg/mL) and kanamycin (40 μg/mL)] and inducers [L-arabinose (2 mg/mL) and lactose (2 mg/mL)]. Naphthalene-2,3-dicarboxaldehyde (NDA) was purchased from AnaSpec Inc., and [^{35}S]cysteine was obtained from Perkin-Elmer.

Plasmids, Cell Cultures, and Purification. Expression plasmids containing *sufS* in pET-21a, *sufE-(His$_6$)* in pBAD (pGSO 165), and *sufABCDSE* in pBAD (pGSO 164)[11] were kindly provided by W. Outten. The plasmid suitable for expression of SufS^{C364A} was generated by QuikChange site mutagenesis (Agilent) using *pET-21A-sufS* as the template. *E. coli* BL21-DE3 cells transformed with *pET-21A-sufS* or pGSO165 were grown at 300 rpm and 37 °C in LB medium with ampicillin. At an OD_{600} of 0.5, cells were induced with lactose and growth was continued for an additional 4 h at 30 °C. *E. coli* CL100 cells transformed with pGSO164 were cultured at 30 °C overnight in LB medium with ampicillin and L-arabinose. All cell cultures were harvested by centrifugation at 8200*g* for 10 min and stored at −20 °C until further use. Frozen cell pellets containing SufS or SufABCDE were resuspended in 25 mM Tris-HCl (pH 8) with 10% glycerol (buffer A), and cell pellets containing SufE were resuspended in buffer A with 0.3 M NaCl. The cell suspensions were lysed with an EmulsiFlex-C5 high-pressure homogenizer. Cell debris was removed by centrifugation at 12800*g* for 30 min. Purification of SufE containing a histidine tag was performed as previously described.[11]

For purification of SufS and SufS^{C364A}, crude extracts were treated with 1% (w/v) streptomycin sulfate followed by centrifugation at 12800*g* for 30 min. The SufS-containing clear supernatant was treated with solid ammonium sulfate until 35 and 55% saturation were reached. The pellet resulting from the 55% saturating concentration of ammonium sulfate obtained after centrifugation at 18000*g* for 20 min was resuspended in an equal volume with buffer A before being loaded onto a Q-sepharose column (1.5 cm × 25 cm) previously equilibrated with buffer A. After the column had been washed with buffer A, proteins were eluted in a linear gradient from 0 to 0.5 M NaCl in the same buffer. Fractions containing SufS were again precipitated with 50% ammonium sulfate (saturating concentration) followed by centrifugation at 18000*g* for 20 min. The pellet was resuspended in 2 mL of buffer A containing 0.15 M NaCl and loaded onto a 2.5 cm × 75 cm Sephacryl S-200 gel filtration column (GE Healthcare) previously equilibrated with the same buffer. Fractions containing SufS were analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE).[17] The protein concentration was determined by the Bradford method using BSA as the standard.[18] The PLP content was analyzed as previously described.[19]

Cell extracts containing SufABCDSE were cleared upon treatment with 1% (w/v) streptomycin sulfate followed by centrifugation. The clear supernatant was loaded into a 1.5 cm × 20 cm Q-sepharose column (GE Healthcare) previously equilibrated with buffer A. After 5 column volume washes with buffer A in 0.1 M NaCl, a linear gradient from 0.1 to 0.7 M

NaCl was employed. Fractions containing the SufBCD complex were combined and precipitated with a 50% saturating concentration of ammonium sulfate. Following centrifugation at 18000*g* for 20 min, the resulting pellet was resuspended in 2 mL of buffer A containing 0.15 M NaCl and loaded onto a 2.5 cm × 75 cm Sephacryl S-200 column (GE Healthcare) previously equilibrated with the same buffer. Fractions containing SufBCD proteins were analyzed by SDS–PAGE,[17] and the protein concentration was quantified using Bradford reagent and bovine serum albumin as the standard.[18] The purified SufBCD complex was assumed to be the $SufBC_2D$ heterotetramer as reported previously.[11]

Cysteine Desulfurase Assay. Cysteine desulfurase activity was determined by quantifying the amount of both products, alanine by derivatization with NDA and sulfide by formation of methylene blue as described by Selbach et al.[16] Unless indicated, the reaction mixtures (800 μL) contained 13.5 nM SufS, 0.5 mM cysteine in 50 mM MOPS (pH 8) buffer, and 2 mM dithiothreitol (DTT), 2 mM tris(2-carboxyethyl)-phosphine hydrochloride (TCEP), 10 mM dithionite (DTN), or 2 mM reduced glutathione (GSH). Rates of sulfide production could not be determined when TCEP was used as the reducing agent because the standard assay is coupled to the formation of methylene blue, which is easily reduced by TCEP. In this work, we have also adapted the indirect high-performance liquid chromatography fluorescence method for quantifying alanine[16] to a 96-well plate reader. This method was used to obtain the pH curve for cysteine desulfurase activity of SufS at pH values ranging from 5 to 8.5, buffering with 50 mM sodium acetate (pH 4.5–5), MES (pH 5–6.8), MOPS (pH 7–8.1), and bicine (pH 8.3–9.5). Reaction mixtures (500 μL) contained 0.45 μM SufS, 2.22 μM SufE, and 2 mM TCEP or DTT. At each time point, 50 μL aliquots were mixed with 5 μL of 10% trichloroacetic acid (TCA). Reaction mixtures were developed with the addition of 200 μL of freshly prepared borate mix [0.61 mM NDA and 6 mM KCN in 115 mM sodium borate (pH 9)]. The solution mix was allowed to react in the dark for 20 min before being read in a flat-bottom microplate (Costar) using a Synergy H1 plate reader (Biotek) with excitation at 390 nm and emission at 440 nm. The fluorescence intensity of each sample was converted into nanomoles of alanine using the slope of an alanine standard curve prepared in the MOPS reaction buffer containing the same concentrations of cysteine and reducing agent used in the assay.

^{35}S Sulfur Transfer Assays. ^{35}S sulfur transfer assays (15 μL) were performed in 50 mM Mops (pH 8) in the presence of 0.05 μM SufS, 0.15 μM SufE, 1.5 μM $SufBC_2D$, 2 mM DTT, and 0.33 μM L-[^{35}S] cysteine. Reaction mixtures were incubated at room temperature for 45 min, quenched with 5 μL of 0.5 M *N*-ethylmaleimide (NEM) for 5 min, and denatured with 10 μL of sample buffer [62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, and 0.001% bromophenol blue]. Each reaction aliquot (15 μL) was analyzed in a nonreducing 20% SDS–PAGE gel. The pulse–chase assays (15 μL) were performed in 50 mM Mops (pH 8) in the presence of 0.02 μM SufS, 0.06 μM SufE, and 2 mM DTT. Reaction 1 was pulsed with 0.4 μM [^{35}S]cysteine for 30 min and then chased with 0.83 mM L-cysteine for an additional 30 min. Reaction 2 was pulsed with 1 mM L-cysteine for 30 min and then chased with 0.32 μM [^{35}S]cysteine for an additional 30 min. Data for time points before and 5 and 30 min after chasing (times 0, 5, and 30, respectively) were collected. The reaction at each time point (5 μL) was quenched with 1 μL of 0.5 M NEM and the mixture denatured with 2 μL of sample buffer. Each reaction aliquot (7 μL) was analyzed in a nonreducing 20% SDS–PAGE gel. All of the ^{35}S labeling was detected by a phosphorimager (Bio-Rad). The density associated with each band was compared to that of the SufS labeling in reaction mixtures containing SufE and [^{35}S]cysteine.

RESULTS

Biphasic Kinetic Profile of the SufS Cysteine Desulfurase Reaction in the Presence of SufE. *E. coli* SufS shows a slow rate of sulfide and alanine formation in the standard cysteine desulfurase assay (Figure 1). Addition of SufE to the reaction mixture enhances the rate of sulfide formation 8–50-fold under reducing conditions.[11,14] However, the mechanism of such activation has not yet been determined. During an initial survey of SufS' activity in the presence of increasing concentrations of SufE, an increase in the baseline levels associated with the slopes of product (sulfide and alanine) versus time was observed (Figure 1B).

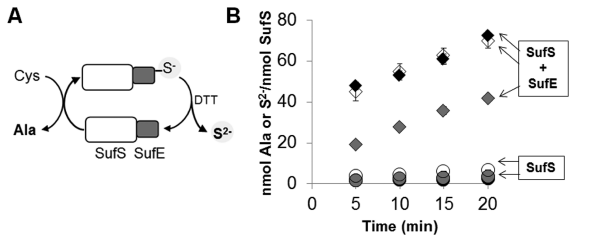


Figure 1. Reaction profile of SufS cysteine desulfurase. (A) Schematic diagram of the SufS–SufE reaction. (B) Product formation of nanomoles of Ala per nanomole of SufS (empty symbols) and nanomoles of sulfide per nanomole of SufS (filled symbols) over time (minutes). Reaction mixtures contained 0.5 mM cysteine, 2 mM DTT, and (●) 0.55 μM SufS or (◆) 28 nM SufS and 0.56 μM SufE. At each time point, 200 μL aliquots were quenched with 20 μL of a 10% TCA solution (empty symbols and black symbols) or with 12.5 μL of 12 M NaOH (gray symbols). Mixtures for reactions terminated with TCA were neutralized with 100 μL of 1 M Tris-HCl (pH 8) (black symbols) or 1 mL of 150 mM sodium borate (pH 8.5) (empty symbols) buffer prior to product quantification. When not visible, error bars are smaller than the symbols.

To investigate the basis of this abnormal profile, the reaction course was tracked by quantifying the alanine formed over short time intervals. Using this approach, a biphasic reaction profile was revealed in assays containing 20 molar equiv of SufE. Under DTT reducing conditions, the slope associated with the first phase of the reaction was 17-fold higher than that displayed in the second phase (Figure 2B and Table 1). The specific activity associated with the first phase was 748 ± 74.2 nmol of Ala min^{-1} mg^{-1}, while that of the second phase was 34.0 ± 6 nmol of Ala min^{-1} mg^{-1}. While the rate of the first phase was not affected by varying the level of SufE in this assay, the duration and amplitude of this phase were proportional to the amount of SufE present in the reaction mixture. Interestingly, the amplitude of the first slope (nanomoles of Ala per nanomole of SufS) was approximately twice the number of moles of SufE present in the reaction mixture. Assays containing 40 equiv of SufE formed 83 ± 17 equiv of Ala in the first phase, while 20 equiv of SufE elicited the formation of 42 ± 8 equiv of Ala.

dx.doi.org/10.1021/bi4001479 | *Biochemistry* 2013, 52, 4089–4096

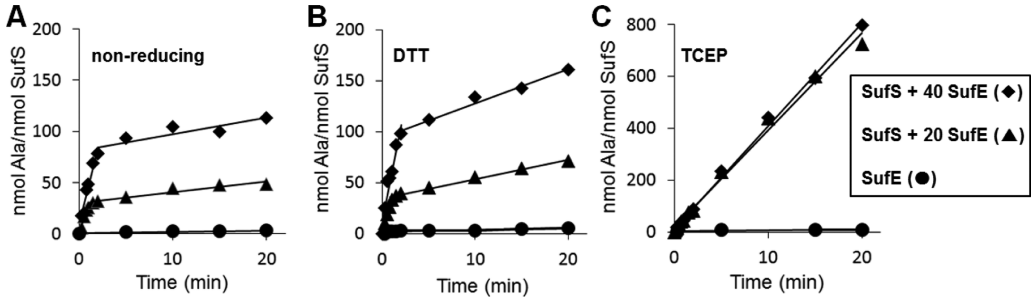


Figure 2. Biphasic kinetic behavior of the SufS−SufE reaction. Cysteine desulfurase activity profile of reaction mixtures containing (●) 0.55 μM SufS, (▲) 28 nM SufS and 0.56 μM SufE, or (◆) 28 nM SufS and 1.12 μM SufE. Reaction mixtures (2 mL) containing 0.5 mM cysteine were assayed under nonreducing conditions (A), in 2 mM DTT (B), or in 2 mM TCEP (C). The profile of alanine formation (nanomoles of Ala produced per nanomole of SufS) was plotted against time (minutes). The graphs display representative reaction profiles from individual experiments of at least three independent assays. The slopes from the linear fit of each reaction phase were used to calculate specific activities listed in Table 1.

Table 1. Effects of Reducing Agents on the Activity of SufS in the Presence of Sulfur Acceptors

				specific activity[a] (nmol of Ala min^{-1} mg^{-1})	
				first phase[b]	second phase[b]
–	SufS			2.9 ± 1.5	
	SufS	with 20SufE		552 ± 48	28.3 ± 4.5
		with 40SufE		604 ± 56	29 ± 5
GSH	SufS	with 20SufE		574 ± 64	58.7 ± 7.7
	SufS	with 40SufE		625 ± 45	59 ± 6.0
DTT	SufS				5.3 ± 0.5
	SufS^{C364A}			ND[c]	
	SufS^{C364A}	with 20SufE		ND[c]	
	SufS	with 5SufE		227 ± 10	19.0 ± 1.4
	SufS	with 20SufE		748 ± 74.2	34.0 ± 6.0
	SufS	with 20SufE^{C51A}		0.5 ± 0.5	
	SufS	with 40SufE		714.8 ± 80	85 ± 25
	SufS	with 5SufE	with 40SufBCD	183 ± 16	
	SufS	with 40SufE	with 40SufBCD	603 ± 3	329 ± 15
	SufS		with 40SufBCD	19 ± 4	
	SufS	with 20SufE		748 ± 74.2	34.0 ± 6.0
TCEP	SufS			11.5 ± 0.5	
	SufS	with 5SufE		533 ± 40	
	SufS	with 20SufE		816 ± 16	
	SufS	with 40SufE		863 ± 59	
	SufS	with 40SufE	with 40SufBCD	642 ± 82	
	SufS		with 40SufBCD	21.5 ± 1.5	

[a]The specific activity was calculated from the linear rate of product formation from at least four time points. [b]The phase was defined as a segment of the overall reaction displaying a linear rate of product formation. [c]Not detected.

The biphasic reaction profile was not limited to the presence of DTT as a reducing agent. Assays performed in the presence of reduced glutathione displayed similar profiles and associated activities for the first and second phases of the reaction (Table 1). Interestingly, cysteine desulfurase reactions under nonreducing conditions (i.e., in the absence of DTT) also showed biphasic alanine release where the rate of the first phase was similar to that observed in the presence of DTT, while the second phase of the reaction displayed a much slower rate (Figure 2A and Table 1). In these reactions, the biphasic behavior of the SufS−SufE reaction in the absence or presence of DTT or glutathione posed a technical challenge for kinetic analysis, as steady-state conditions were obtained only during the second phase of the reaction. The transition to the second phase could be associated with the accumulation of persulfurated/polysulfurated forms of SufE (SufE-S_n^-) which would then participate as a poor substrate in the reaction. In support of this proposal, the cysteine desulfurase reaction in the presence of [^{35}S]cysteine showed transfer of ^{35}S to SufE despite the presence of DTT (Figure 3A, lane 2, and ref 11). Quantification of the ^{35}S bands indicated that the relative radioactivity of SufE was 3 times higher than that of SufS, in a reaction mixture containing a 3-fold molar excess of SufS (Figure S4 of the Supporting Information), indicating that both proteins retained a similar number of sulfur modifications. Interestingly, pulse−chase experiments show that such a modification occurs during the initial phase of the reaction and is not eliminated during turnover (Figure 3B).

dx.doi.org/10.1021/bi4001479 | Biochemistry 2013, 52, 4089−4096

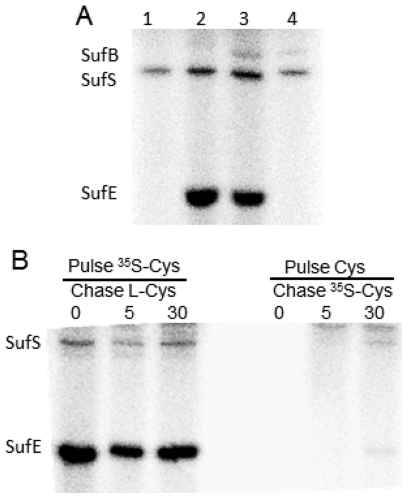


Figure 3. Protected persulfide sulfur transfer from SufS to SufE. (A) Phosphorimager depiction of a nonreducing SDS−PAGE gel containing SufS (lane 1), SufS and SufE (lane 2), SufS, SufE, and $SufBC_2D$ (lane 3), and SufS and SufBCD (lane 4). Each lane contained 0.37 pmol of SufS, 1.1 pmol of SufE, and 11 pmol of $SufBC_2D$. (B) Phosphorimager depiction of the pulse−chase experiment for SufS (0.16 pmol) and SufE (0.49 pmol): (left) reaction mixtures incubated with [^{35}S]cysteine for 30 min (pulse) and chased with cysteine for 5 and 30 min and (right) reaction mixtures incubated with cysteine for 30 min (pulse) and chased with [^{35}S]cysteine for 5 and 30 min. The relative radioactivity associated with each band is reported in Figure S4 of the Supporting Information. In assays containing a 3-fold molar excess of SufE, the bands associated with SufE were nearly 3 times more intense than those associated with SufS.

Accumulation of SufE-S_n^- under DTT reducing conditions also helps to explain the differences in the baseline of alanine and sulfide assays (Figure 1B). When using the standard protocol for sulfide quantification, reactions were quenched upon addition of the NaOH solution, the first reagent used for the formation of methylene blue. This method allows quantification of only free sulfide species at each time point of the reaction [Figure 1B (gray diamonds)]. To quantify the total sulfur mobilized by cysteine (free sulfide plus persulfide/polysulfide associated with protein), reactions were terminated upon addition of TCA to denature the proteins and then aliquots were neutralized to allow reduction of persulfide/polysulfide prior to formation of methylene blue. Following this procedure, the profile of sulfide production mirrored that of alanine formation [Figure 1B (filled and empty diamonds)], where the number of moles of alanine at each time point was identical to the number of moles of total sulfur (free S^{2-} plus SufE-S_n^-).

TCEP Regenerates SufE at the End of Each Catalytic Cycle. The biphasic behavior of the SufS and SufE reaction suggested that the persulfide bond was shielded from DTT or the protein environment surrounding the persulfide bond could offer chemical protection by lowering the redox potential of this bond. To test the second hypothesis, we first followed the cysteine desulfurase reaction in the presence of the larger but stronger reducing agent, TCEP. As shown in Figure 2C, when TCEP was used as a reducing agent the reaction displayed linear product formation with an associated rate (Figure 2A) similar to that observed in the first phase of the reaction in the absence or presence of DTT (Figure 2B). Second, we analyzed the sulfur covalently bound to SufS−SufE samples (as persulfide or polysulfide forms), after addition of cysteine (Table S1 of the Supporting Information). These analyses indicated that SufE retains sulfur modification (approximately 0.75 ± 0.13 sulfur/monomer) after incubation with SufS and cysteine under DTT reducing conditions. These results were distinct from those of assays performed in the presence TCEP or dithionite, where only 0.1 ± 0.08 and 0.05 ± 0.005 sulfur/SufE were detected (Table S1 of the Supporting Information).

Steady-State Kinetic Analysis of the SufS−SufE Reaction. The sustained linear steady-state kinetics of alanine formation in the presence of TCEP allowed us to determine the rate constants for the Cys:SufE sulfurtransferase reaction of SufS. The substrate saturation curve indicated the high degree of selectivity of the enzyme for its sulfur acceptor substrate SufE [Figure 4 (diamonds)] with an associated K_M (0.87 μM)

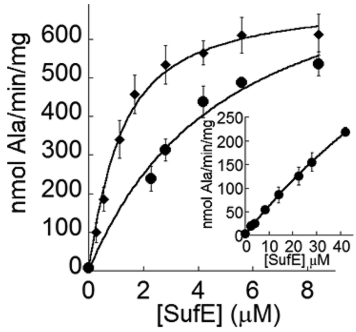


Figure 4. Substrate saturation curves catalyzed by SufS. Assays were performed in 50 mM Mops (pH 8), 0.5 mM cysteine, 0.55 μM SufS, and varying concentrations of SufE (0.28−8.4 μM). The rate of alanine formation (nanomoles of Ala per minute per milligram) was calculated from the linear slopes of at least three time points for reaction using 2 mM TCEP (◆) or 2 mM DTT (●). The lines are the best fit for the equation for high-affinity substrates: $v = v_{max}/(2E_t) \times \{E_t + K_M + S_t - [(E_t + K_M + S_t)^2 - 4S_tE_t]\}^{1/2}$, where v is the rate of alanine formation, E_t is the enzyme concentration, and S_t is the substrate concentration added to the assay.[23] In assays measuring the first phase of DTT reducing reactions, contributions of the second phase of the reaction were negligible within this substrate concentration range. The velocity associated with the first phase of DTT reducing reactions resulted in an apparent kinetic constant (v_{max}) of 877 ± 128 nmol of Ala min^{-1} mg^{-1} and a K_M of 4.6 ± 1.4 μM, while constants determined under TCEP reducing conditions were 703 ± 25 nmol of Ala min^{-1} mg^{-1} (v_{max}) and 0.87 ± 0.12 μM (K_M). The inset shows the rates of Ala formation in the presence of increasing amounts of SufE under DTT reducing conditions during the second phase of the reaction (e.g., at time points 10, 15, and 20 min). The line is the best fit of the Michaelis−Menten equation. No substrate saturation was observed up to 40 μM SufE.

similar to that reported for *B. subtilis* SufU sulfur acceptor protein (3 μM).[16] For comparison, the activity profile of alanine formation in the presence of DTT was also determined at increasing concentrations of SufE [Figure 4 (circles)]. The activity associated with the first phase and the second phase [Figure 4 (inset)] was plotted against the SufE concentration. Because there was no indication of saturation, the expected K_M for SufE in the second phase of the reaction is assumed to be much higher than that calculated for the first phase of the reaction. This observation supports the idea that SufE-S_n^- can also serve as a substrate, although with limited reactivity.

The proposed kinetic mechanism of the Cys:SufS sulfurtransferase reaction of SufS resulting in the persulfide sulfur transfer to the $SufE^{C51}$ substrate could follow two possible schemes. In the first model, the SufS reaction could proceed

dx.doi.org/10.1021/bi4001479 | *Biochemistry* 2013, 52, 4089−4096

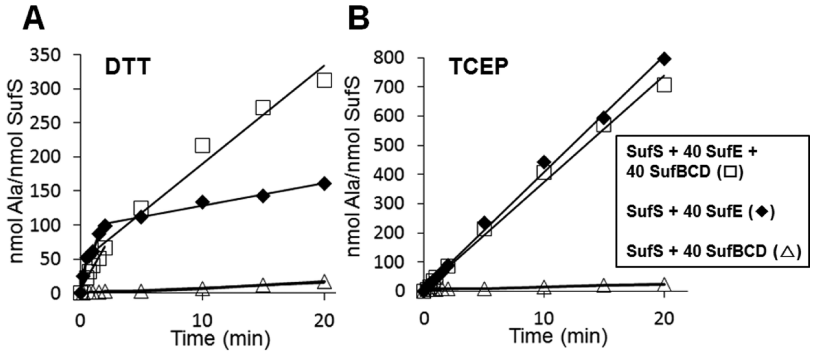


Figure 5. Effect of SufBCD on the SufS−SufE cysteine desulfurase reaction. Cysteine desulfurase activity profile of a reaction mixture containing (△) 28 nM SufS and 1.12 μM SufBCD complex, (◆) 28 nM SufS and 1.12 μM SufE, and (□) 28 nM SufS, 1.12 μM SufE, and 1.12 μM SufBCD. Reaction mixtures (2 mL) containing 0.5 mM cysteine were assayed using 2 mM DTT (A) or 2 mM TCEP (B). The rates of alanine formation (nanomoles of Ala produced per nanomole of SufS) were plotted vs time (minutes). The graphs display representative reaction profiles from individual experiments of at least three independent assays. The slopes from the linear fit of each reaction phase were used to calculate specific activities listed in Table 1.

through formation of a ternary complex, bypassing the formation of a covalent enzyme intermediate. In this case, SufS would promote a one-step sulfur transfer reaction directly from cysteine to SufE. In the second model, the reaction in the presence of SufE would still proceed through formation of a covalent enzyme intermediate at the active site cysteine followed by the persulfide sulfur transfer to the $SufE^{C51}$ substrate. Results described here support the latter proposal because the $SufS^{C364A}$ variant lacks activity in the presence and absence of SufE (Table 1) and S labeling is also observed on SufS when the enzyme is incubated with SufE and [^{35}S]cysteine (Figure 3). In addition, the lack of activity of $SufE^{C51A}$ when it serves as a substrate of this reaction (Table 1) along with the parallel lines on double-reciprocal plots of cysteine saturation curves (Figure S1 of the Supporting Information) provides support for a ping-pong mechanism. When using 10 equiv of SufE (10 μM), the K_{Mcys} was determined to be 14 ± 1.7 μM. However, at lower SufE concentrations, an accurate assignment of the kinetic parameters was not made as the K_{Mcys} is around or below the detection limits of the assay (LOD = 0.1 pmol of Ala). The pH−activity profile of SufS in the absence and presence of SufE (5 molar equiv) indicated that under assay conditions (pH 7.4−8), the rate of the reaction was dependent on SufE, suggesting that the thiolated form of SufE likely promotes the nucleophilic attack on the terminal persulfide sulfur of SufS. Unexpectedly, significant activity was also observed in a lower pH range (5.5−6.6), which was independent of SufE (Figure S2 of the Supporting Information). The basis of this activity profile was not further investigated in this work; however, it is worth noting that *B. subtilis* SufS exhibits a similar profile (P. C. Dos Santos and P. K. Pradhan, unpublished results). Analysis of the crystal structures of *E. coli* and *Synechocystis* SufS shows the presence of three conserved residues (His^{123}, His^{225}, and Gln^{203} in the *E. coli* SufS sequence) in addition to Cys^{364}. These four residues are in the proximity of the PLP-Lys Schiff base that could serve as active participants in these ionization events.

Sequential Sulfur Transfer from SufS to SufE to SufBCD. The formation of Fe−S clusters under oxidative stress conditions is proposed to occur on the scaffold SufB protein when it was in complex with SufC and/or SufD.[20] Previous studies have shown that addition of the SufBCD complex to cysteine desulfurase reaction mixtures containing SufS and SufE further enhances the turnover rate of sulfide production, the mechanism of which remains unexplored. To gain further insight into the Suf-dependent pathway of sulfur trafficking, we have explored the effect of SufBCD in the SufS reaction. Under DTT reducing conditions, the SufS−SufE reaction in the presence of SufBCD still displayed biphasic behavior; however, the activity associated with the second phase of the reaction was enhanced 3-fold, while causing modest inhibition of the first phase of the reaction (Figure 5A and Table 1). Furthermore, at subsaturating SufE concentrations (1:5 SufS:SufE ratio), the presence of SufBCD enhanced nearly 10-fold the activity of SufS−SufE reactions. The SufS:5SufE:40SufBCD reaction profile was linear, indicating that, under these conditions, the rate of the reaction is not limited by the regeneration of SufE. On the basis of these results, the effect of SufBCD in enhancing the rates of alanine formation by the activity of SufS could be explained by at least three distinct mechanisms. (i) SufBCD directly interacts with SufS activating the enzyme. (ii) The SufBCD complex directly interacts with SufE, leading to conformational changes on the protein that would deprotect the SufE persulfide sulfur making it susceptible to reductive cleavage. (iii) The SufBCD complex serves as a sulfur acceptor of SufE.

Under TCEP reducing conditions, reaction mixtures containing SufS, SufE, and SufBCD also displayed a linear formation of alanine with associated rates similar to those calculated for the first phase of the DTT reducing reactions. The presence of the SufBCD complex caused an only modest reduction in activity (Figure 5B), suggesting that TCEP can bypass the sulfur transfer step from SufE to SufB, but not that from SufS to SufE.

Labeling experiments using excess [^{35}S]cysteine indicated that the presence of the SufBCD complex does not affect the ^{35}S modification of SufE. Reaction mixtures containing SufS, SufE, and SufBCD were incubated under turnover conditions for 30 min in the presence of [^{35}S]cysteine and 2 mM DTT before the reactions were quenched with 50 mM NEM and analyzed via nonreducing SDS−PAGE (Figure 3). In these experiments, DTT was not effective in cleaving the persulfide on SufS or SufE in its active conformation (Figure 3, lanes 1 and 2). However, ^{35}S labeling was not observed when DTT was added after SDS denaturation, thus indicating that SufE's structural fold provides chemical protection of the persulfide bond (data not shown). In the same experiment, modest labeling of SufB was only observed when reactions were

dx.doi.org/10.1021/bi4001479 | *Biochemistry* 2013, 52, 4089−4096

performed in the presence of 2 mM DTT (Figure 3, lane 3); however, no labeling was detected when reactions were performed at higher DTT concentrations (e.g., >10 mM) (data not shown). The ability of the SufS to display limited capacity to transfer sulfur to SufB, as observed in labeling experiments, is in agreement with the enhancement of SufS activity in the presence of SufBCD (Table 1). These results provide support to the model involving a sequential and protected sulfur relay system from SufS to SufE to SufBCD, where the flux of sulfur transfer is regulated on the basis of the demand by the final acceptor, SufB (Figure 6).

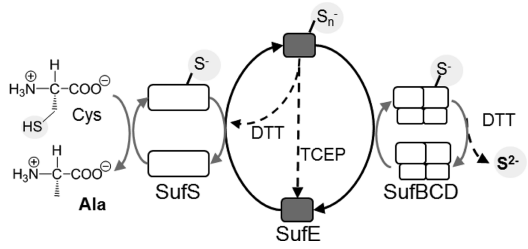


Figure 6. Proposed protected sulfur transfer pathway of the *E. coli* Suf system. Solid arrows depict the flow of sulfur transfer from cysteine to the final acceptor SufBCD complex. Dashed arrows depict the reduction of persulfurated/polysulfurated proteins by reductants present in the *in vitro* assay.

DISCUSSION

Model for Protected Persulfide Sulfur Transfer from SufS to SufE. On the basis of the profile of the SufS and SufE reaction, we hypothesized that the completion of the first phase of the reaction could be associated with the depletion of the active form of SufE. In this case, the initial rate of first phase reflects the reaction of initial sulfur transfer from SufS to SufE resulting in accumulation of SufE-S_n^-. In this proposed model, the persulfide sulfur would be protected from reductive cleavage by DTT, leading to accumulation of more than one sulfur atom onto the $SufE^{Cys51}$ residue. Following the formation of SufE-S_n^-, the reaction transitioned to a second phase, displaying a slow turnover rate. In this latter case, SufE-S_n^- could also act as a sulfur acceptor, thus expanding the length of the polysulfide chain that could then be susceptible to reduction by DTT. In reactions displaying a biphasic behavior, the first phase was associated with the formation of nearly 2 equiv of Ala/SufE present in the reaction, suggesting that each SufE could receive up to two sulfurs in this phase. The subsequent cleavage of the second sulfur by DTT could represent a slow step, leading the reaction to the second phase. Further evidence of this proposal was displayed by the ability of SufE to retain approximately one sulfur atom in the presence of 2 mM DTT as shown by sulfur analysis of SufE after cysteine desulfurase reactions.

The use of the stronger reducing agent, TCEP, on the other hand, generated a linear profile of alanine formation, preventing the reaction from entering the second phase. Under these conditions, the maximal velocity for this reaction was higher than previously reported values for this enzyme.[8,21] The effect of TCEP on the catalytic cycle of SufE can be attributed to the regeneration of the sulfur acceptor at the end of each turnover. The occurrence of two phases of the cysteine desulfurase reaction under standard *in vitro* conditions challenges the assessment of enzyme activity through single-time, end point assays. Without multiple samples following the time course, these approaches obscure the reaction profile and lead to differences in the calculated rates of product formation. In addition, the SufS–SufE reaction profile could also explain the abnormal kinetic behavior of this enzyme that may lead to differences in activity levels reported for this enzyme. In our studies, no enzyme inhibition was observed when TCEP was used as the reducing agent.

Mass spectrometry analysis of SufE after incubation with SufS and cysteine led to accumulation of two to four sulfur atoms associated with SufE,[14] which was in agreement with our sulfur analysis of SufE after the cysteine desulfurase reaction under nonreducing conditions (Table S1 of the Supporting Information). The crystal structure of SufE indicates that the Cys^{51} sulfur acceptor site is located in a hydrophobic cavity shielded from the solvent.[22] Here, we show that the rate of sulfur transfer is dependent on the sulfurization status of SufE, which has an impact on the overall SufS reaction rate (i.e., SufE is a better substrate than SufE-S_n). Presumably, under conditions of high demand for Fe–S cluster biogenesis, free forms of SufBC or SufBCD would dictate the flux of sulfur trafficking from SufS to SufE to SufB. On the other hand, when SufB is not available, a buildup of SufE-S_n would downregulate the catalytic reactivity of SufS, preventing a futile cycle. In this model, the rate of sulfur transfer is adjusted to the demand of the final acceptor. It is possible that the use of an intermediate carrier is important for guaranteeing the protection of a labile persulfide from the action of reactive oxygen species encountered under conditions faced by the Suf system. The reaction profile of SufS in the presence of sulfur acceptors described in this report provides the kinetic basis for activity enhancement by SufE and SufBCD. This concerted sulfur transfer path assures protection of reaction intermediates at a controlled flux to meet cellular demands encountered under conditions detrimental to thiol chemistry and Fe–S cluster metabolism.

ASSOCIATED CONTENT

Supporting Information

Analysis of sulfur associated with SufS–SufE samples after incubation with cysteine had been quantified by methylene blue formation (Table S1) and by densitometry of ^{35}S radioactive gel bands (Figure 4S), additional kinetic analysis of the SufS cysteine desulfurase reaction that includes the cysteine substrate saturation curves of SufS in the presence of fixed SufE concentrations (Figure S1), pH–activity profile of SufS and SufS–SufE reaction (Figure S2), and kinetic profile of SufS DTT reducing reaction mixtures containing subsaturating concentrations of SufE in the absence and presence of SufBCD (Figure S3). This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: dossanpc@wfu.edu, 336-758-3144 (phone), 336-758-4656 (fax).

Funding

This research was funded by the National Science Foundation (MCB-1054623).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Dr. W. F. Outten for the generous gift of expression plasmids containing *sufS*, *sufE*, and *sufABCDSE* and Rebecca Alexander and Leslie Poole for critical reading of the manuscript.

■ REFERENCES

(1) Py, B., and Barras, F. (2010) Building Fe-S proteins: Bacterial strategies. *Nat. Rev. Microbiol. 8*, 436−446.
(2) Mueller, E. G. (2006) Trafficking in persulfides: Delivering sulfur in biosynthetic pathways. *Nat. Chem. Biol. 2*, 185−194.
(3) Behshad, E., and Bollinger, J. M., Jr. (2009) Kinetic analysis of cysteine desulfurase CD0387 from *Synechocystis* sp. PCC 6803: Formation of the persulfide intermediate. *Biochemistry 48*, 12014−12023.
(4) Shi, R., Proteau, A., Villarroya, M., Moukadiri, I., Zhang, L., Trempe, J. F., Matte, A., Armengod, M. E., and Cygler, M. (2010) Structural basis for Fe-S cluster assembly and tRNA thiolation mediated by IscS protein-protein interactions. *PLoS Biol. 8*, e1000354.
(5) Hidese, R., Mihara, H., and Esaki, N. (2011) Bacterial cysteine desulfurases: Versatile key players in biosynthetic pathways of sulfur-containing biofactors. *Appl. Microbiol. Biotechnol. 91*, 47−61.
(6) Mihara, H., and Esaki, N. (2002) Bacterial cysteine desulfurases: Their function and mechanisms. *Appl. Microbiol. Biotechnol. 60*, 12−23.
(7) Kato, S., Mihara, H., Kurihara, T., Takahashi, Y., Tokumoto, U., Yoshimura, T., and Esaki, N. (2002) Cys-328 of IscS and Cys-63 of IscU are the sites of disulfide bridge formation in a covalently bound IscS/IscU complex: Implications for the mechanism of iron-sulfur cluster assembly. *Proc. Natl. Acad. Sci. U.S.A. 99*, 5948−5952.
(8) Dai, Y., and Outten, F. W. (2012) The *E. coli* SufS-SufE sulfur transfer system is more resistant to oxidative stress than IscS-IscU. *FEBS Lett. 586*, 4016−4022.
(9) Tirupati, B., Vey, J. L., Drennan, C. L., and Bollinger, J. M., Jr. (2004) Kinetic and structural characterization of Slr0077/SufS, the essential cysteine desulfurase from *Synechocystis* sp. PCC 6803. *Biochemistry 43*, 12210−12219.
(10) Mihara, H., Fujii, T., Kato, S., Kurihara, T., Hata, Y., and Esaki, N. (2002) Structure of external aldimine of *Escherichia coli* CsdB, an IscS/NifS homolog: Implications for its specificity toward selenocysteine. *J. Biochem. 131*, 679−685.
(11) Outten, F. W., Wood, M. J., Munoz, F. M., and Storz, G. (2003) The SufE protein and the SufBCD complex enhance SufS cysteine desulfurase activity as part of a sulfur transfer pathway for Fe-S cluster assembly in *Escherichia coli*. *J. Biol. Chem. 278*, 45713−45719.
(12) Trotter, V., Vinella, D., Loiseau, L., Ollagnier de Choudens, S., Fontecave, M., and Barras, F. (2009) The CsdA cysteine desulphurase promotes Fe/S biogenesis by recruiting Suf components and participates to a new sulphur transfer pathway by recruiting CsdL (ex-YgdL), a ubiquitin-modifying-like protein. *Mol. Microbiol. 74*, 1527−1542.
(13) Loiseau, L., Ollagnier-de-Choudens, S., Nachin, L., Fontecave, M., and Barras, F. (2003) Biogenesis of Fe-S cluster by the bacterial Suf system: SufS and SufE form a new type of cysteine desulfurase. *J. Biol. Chem. 278*, 38352−38359.
(14) Ollagnier-de-Choudens, S., Lascoux, D., Loiseau, L., Barras, F., Forest, E., and Fontecave, M. (2003) Mechanistic studies of the SufS-SufE cysteine desulfurase: Evidence for sulfur transfer from SufS to SufE. *FEBS Lett. 555*, 263−267.
(15) Layer, G., Gaddam, S. A., Ayala-Castro, C. N., Ollagnier-de Choudens, S., Lascoux, D., Fontecave, M., and Outten, F. W. (2007) SufE Transfers Sulfur from SufS to SufB for Iron-Sulfur Cluster Assembly. *J. Biol. Chem. 282*, 13342−13350.
(16) Selbach, B., Earles, E., and Dos Santos, P. C. (2010) Kinetic analysis of the bisubstrate cysteine desulfurase SufS from *Bacillus subtilis*. *Biochemistry 49*, 8794−8802.
(17) Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature 227*, 680−685.
(18) Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem. 72*, 248−254.
(19) Wada, H., and Snell, E. E. (1962) Enzymatic transamination of pyridoxamine. I. With oxaloacetate and α-ketoglutarate. *J. Biol. Chem. 237*, 127−32.
(20) Wollers, S., Layer, G., Garcia-Serres, R., Signore, L., Clemancey, M., Latour, J. M., Fontecave, M., and Ollagnier de Choudens, S. (2010) Iron-sulfur (Fe-S) cluster assembly: The SufBCD complex is a new type of Fe-S scaffold with a flavin redox cofactor. *J. Biol. Chem. 285*, 23331−23341.
(21) Mihara, H., Kurihara, T., Yoshimura, T., and Esaki, N. (2000) Kinetic and mutational studies of three NifS homologs from *Escherichia coli*: Mechanistic difference between L-cysteine desulfurase and L-selenocysteine lyase reactions. *J. Biochem. 127*, 559−567.
(22) Goldsmith-Fischman, S., Kuzin, A., Edstrom, W. C., Benach, J., Shastry, R., Xiao, R., Acton, T. B., Honig, B., Montelione, G. T., and Hunt, J. F. (2004) The SufE sulfur-acceptor protein contains a conserved core structure that mediates interdomain interactions in a variety of redox protein complexes. *J. Mol. Biol. 344*, 549−565.
(23) Henderson, P. J. (1973) Steady-State Enzyme Kinetics with High Affinity Substrates or Inhibitors: A statistical treatment of dose-response curves. *Biochem. J. 135*, 101−107.